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Does Environmental Enrichment Exposure Prior to Injury Influence Biomarkers Associated with Chronic Stage TBI?

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Interim Report

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14. ABSTRACT
Environmental Enrichment (EE) is an innovative technique shown to improve cognitive and functional outcomes, and elevate key biomarkers following traumatic brain injury (TBI). Our study was designed to determine if exposure to EE prior to injury is associated with elevated levels of brain-derived neurotrophic factor (BDNF) and other protective biomolecules during the chronic stage of TBI. To examine the effects of EE before injury, adult male rats were exposed to an enriched environment for 15 days followed by a moderate medial prefrontal cortex injury via controlled cortical impact. After recovery, animals were behaviorally tested for 33 days followed by sacrifice to allow for sample collection and analysis. Results indicated that EE applied before TBI did elevate trkB receptor mRNA levels compared to standard housed animals; however BDNF and other biomarker levels during the chronic stage of TBI were not significantly different. The data suggests that EE exposure prior to TBI has neuroprotective tendencies during the chronic stage; however BDNF and other biomarkers associated with improved performance are not elevated to reflect this mechanism during the timeframe examined.

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1.0   OVERVIEW

Environmental Enrichment (EE) is an innovative technique shown to improve cognitive and functional outcomes, and elevate key biomarkers following traumatic brain injury (TBI). Our study was designed to determine if exposure to EE prior to injury is associated with elevated levels of brain-derived neurotrophic factor (BDNF) and other protective biomolecules during the chronic stage of TBI. To examine the effects of EE before injury, adult male rats were exposed to an enriched environment for 15 days followed by a moderate medial prefrontal cortex injury via controlled cortical impact. After recovery, animals were behaviorally tested for 33 days followed by sacrifice to allow for sample collection and analysis. Results indicated that EE applied before TBI did elevate trkB receptor mRNA levels compared to standard housed animals; however BDNF and other biomarker levels during the chronic stage of TBI were not significantly different. The data suggests that EE exposure prior to TBI has neuroprotective tendencies during the chronic stage; however BDNF and other biomarkers associated with improved performance are not elevated to reflect this mechanism during the timeframe examined.

2.0   INTRODUCTION

Traumatic brain injury (TBI) is a costly, devastating condition which can generate long-lasting social and occupational disabilities in behavior, memory, and executive function (Dawson et al., 1995). Each stage (acute, sub-acute and chronic) of injury is categorized by key cellular, biochemical and physiological changes; however the chronic stage is the longest and most damaging period. This stage is characterized by inflammatory responses, breakdown of the blood brain barrier (BBB), delayed cell death, edema and swelling, and hippocampal damage resulting in deficits in learning and memory (Colicos et al., 1996; Kim, J., 2006; Laurer et al., 2001). However; the chronic stage may be influenced if appropriate measures are taken prior to or following TBI, leading to research focused on preventative measures, such as environmental enrichment.

Environmental enrichment (EE) is a complex sensory-motor stimulation that provides rodents with an increased opportunity for physical exercise, various learning experiences, and social interactions which may result in a variety of neuroplastic changes in the brain (Hebb, D., 1947). Enrichment is shown to improve cognitive performance, neuronal plasticity, memory performance and motor skills during chronic stage TBI in rodents when applied pre- or post-injury (Hebb, D., 1947; Johnson et al., 2012; Will et al., 2003). Other neuroprotective tendencies induced by enrichment include the initiation of cell proliferation, increased messenger RNA and protein synthesis, and neurogenesis in the hippocampus (Rosenzweig et al., 1996; Diamond et al., 1964; Kempermann et al., 1997; Sozda et al., 2010; Passineau et al., 2001; Hamm et al., 1996) caused by modulated expression of neuroprotective biomolecules and neurotrophic factors, specifically brain-derived neurotrophic factor (BDNF) (Piehl et al., 2001; Lee et al., 2008; Huang et al., 2001; Larsson et al., 2002; Derksen et al., 2007; Khan et al., 2011).

Brain-derived neurotrophic factor is associated with learning and memory, is effective in promoting the survival of neurons following TBI and is upregulated with the application of

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enrichment (Grundy et al., 2000; Kohara et al., 2001). BDNF is an activity-dependent modulator of neuronal structure that aids in the regulation of survival and differentiation of neurons (Bramham et al., 2005). BDNF binds to a tyrosine kinase family receptor, trkB, which augments its ability to regulate neuronal proliferation and survival, axonal and dendritic growth, synapse formation and function and repair of damaged neurons (Huang and Reichardt, 2001). In addition, BDNF increases the activity of free radical scavengers and can therefore protect neurons against the damage induced by free radicals (Ang et al., 2007). Previous work shows elevations in BDNF expression in the hippocampus 24-72h following injury (Hicks et al., 1997); however EE exposure post-TBI maintained normal levels of BDNF when observed 16 days after insult (Hicks et. al., 2002). Other research provides evidence of elevated levels of BDNF during recovery (14 days) from TBI (Griesbach et al., 2004) and pre-injury EE exposure (Johnson et al., 2012) has been associated with improved performance on memory tasks in rodents during chronic stage TBI, illustrating the long-term influence EE and neurotrophic factors have on cognitive performance. As shown above, research focusing on neuroprotective biomarkers during chronic stage TBI has demonstrated varying results (Griesbach et al., 2004; Hicks et al., 2002; Hicks et al., 1997; Ickes et al., 2000, Zhao et al., 2000) opening the need for continued research in mechanisms associated with long-term improved function following injury. From these studies, it has been determined that by enhancing BDNF via EE, the brain may be able to augment its synaptic and neuronal connections, providing added neuroprotection against the detriments of chronic stage TBI.

Another neuroprotective biomarker associated with TBI and EE is cortisol. Following insult, the hypothalamic-pituitary-adrenal (HPA) axis is activated and depending on the severity of the injury, can induce a loss of homeostasis. This change to the HPA axis can cause an elevated amount of glucocorticoids (GCs), such as cortisol, to remain in the central nervous system leading to numerous detrimental effects in executive function and eventual cell death (Grundy et al., 2001, Sapolsky, 1990). EE may counteract some of the damaging effects of stress by rendering the HPA axis response more adaptive and efficient through modulation of the synthesis and secretion of GCs to induce physiological changes, restoring homeostasis. Several studies report higher baseline levels of corticosterone in enriched animals (de Jong et al., 2000; Benaroya-Milshtein et al., 2004; Moncek et al., 2004). High baseline corticosterone levels may be related to the continual mild stress induced by repetitive exposure to new objects (Benaroya-Milshtein et al., 2004). The increased baseline corticosterone coupled with reduced stressor-induce corticosterone could lead to neuroprotective tendencies during chronic stage TBI (Larsson et al., 2002; Benaroya-Milshtein et al., 2004).

Few studies have examined the influence of EE on neuroprotective biomarkers when applied pre-TBI. Recent research completed by Johnson et al. (2012) determined that EE applied prior to injury caused improved performance on behavioral memory tasks in rodents during the chronic stage of TBI. However, understanding the long-term benefits that EE may induce biochemically if applied before injury has not been explored. This research focused on the effects of pre-enrichment on neuroprotective biomarkers during the chronic stage of TBI. Understanding how this mitigation strategy can affect long-term outcomes in cognition and performance is vital for preventing enduring deficits to patients who experience a TBI and identifying targets for treatment and intervention.

3.0   METHODS

3.1 Animals
Animals used in this study were male Sprague Dawley rats approximately 90 days of age from Charles River Laboratories. Upon arrival, animals were separated into various housing conditions in a room with stable temperature (25°C) and a 12 hour light/dark cycle. The animals were provided *ad libitum* access to food and water and handled for 5 min/day or more depending on their housing condition. Animals were weighed on a daily basis and were given 7 days to acclimate before commencement.

3.2 Housing Conditions
Environmental enrichment (EE-TBI) rats were housed 8 per cage in a large wire mesh cage (~1m^3) with a variety of items such as a running wheel, plastic tubes, ladders, ropes and mirrors for 15 days before TBI (Figure 1). Each day the toys were rearranged at feeding and twice weekly the items were replaced by new toys in order to provide the animals with novel stimuli. Enriched animals also received acrobatic training and were exposed to olfactory stimulation twice a day for 10 days (two 5-day blocks) following the acclimation period. The EE cages were kept out of sight from the standard and control animal cages using wall dividers. Enriched animals were subjected to a medial pre-frontal TBI following the 15-days of EE housing.

Figure 1: Traditional Enriched Housing. Animal environmental enrichment includes group housing in large cages with exposure to novel objects.

Standard (ST-TBI) and control (Sham) animals were housed 2 per standard polycarbonate shoebox cage for 15 days before TBI. These animals did not receive contact with other animals or additional training and were handled with minimal contact (5min/day) during routine cage changing. Standard animals were subjected to a medial pre-frontal TBI following the 15-days of standard housing. The control group consisted of animals which underwent anesthesia and a craniotomy but did not receive an injury.

3.3 Controlled Cortical Impact
Enriched and ST-TBI animals were subjected to TBI via a controlled cortical impact (CCI) device. Animals were anesthetized with isoflurane (5% induction, 2% maintenance) and secured with their heads fixed in a horizontal position. Blood saturation of peripheral oxygen (SpO$_2$) and body temperature were closely monitored and maintained throughout the surgery.   A 6mm craniotomy was performed 5mm anterior to bregma and the bone segment removed was discarded.   An electrical contusion impactor with a velocity of 2.25m/sec and a

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depth of 3mm was used to inflict the TBI at the prefrontal cortex. Following the procedure, the scalp was sutured with 7mm surgical staples. Sham controls were anesthetized and received a craniotomy but not a CCI. After surgery and recovery, animals were returned to their original cages and cage mates. The EE group no longer received additional stimuli or enrichment. By discontinuing EE at this stage, data would reflect the effects of enrichment *prior* to injury only.

3.4 Blood Collection
Non-stressed blood collections were taken the morning before enrichment started (baseline collection) and again after the 15 days of housing (basal collection). On the morning of sacrifice, animals were subjected to a 20 minute restraint stress, with blood collected immediately after the restraint. The stress challenge was conducted between the hours of 0700 and 0900 to control for diurnal fluctuations in hypothalamic-pituitary-adrenal (HPA) axis activity. All blood samples were taken by tail clip for measurement of plasma corticosterone.

3.5 Tissue Collection
Animals were sacrificed 33 days post-injury. They were briefly anesthetized with 5% isoflurane until a toe pinching test received no reaction and decapitated via guillotine. The brains were removed and split in half saggitally. Alternating left and right sides were kept intact and flash frozen in isopentane. For *in- situ* hybridization analysis, coronal sections of the brain hemispheres (14 µm) through the prefrontal cortex and hippocampus were cut on a cryostat and stored at -20˚C. The contralateral side was dissected for Enzyme-Linked Immunosorbent Assay (ELISA) and Western Blot analysis techniques and stored at -80˚C until analyzed.

3.6 Biochemical Analysis
Plasma corticosterone levels were measured using a commercial 125I radioimmunoassay kit (MP Biomedicals, LLC, Orangeburg, NY). Briefly, samples were diluted 1:20 using diluent, and all samples were added to appropriately labeled 10x75mm glass test tubes. Corticosterone 125I was placed in all tubes followed by anti-corticosterone. Samples were then vortexed and incubated at room temperature for 2 hours. Following incubation, 0.5mL precipitant solution was placed in each tube and samples were again vortexed. Samples were centrifuged at 2400rpm for 15 minutes and supernatant was decanted. The precipitate was then counted using a gamma counter and concentration was calculated.

For analysis of proteins using Western Blot and ELISA, frozen tissue was homogenized in sucrose buffer, and then centrifuged for 8 minutes at 4°C and 6000xg. The supernatant of each sample was transferred to a fresh microcentrifuge tube and a protein assay was performed using the Thermo Scientific Pierce BCA Assay Kit to determine total protein.

To complete the western blot analysis of MAP2 and Synaptophysin the following procedures were performed. Briefly, protein supernatants were normalized (6.5µg/100mL), heated in a 65ºC water bath, and then loaded into gels and run in duplicate at 150V for 1.5 hours. Gels were transferred onto polyvinylidene difluoride (PVDF) membranes, and washed with phosphate buffered saline with 0.05% tween (PBS-T). The membranes were blocked with 5% blotto, then incubated with antibodies for MAP2 [Abcam Cambridge, MA; 11268, 1:50,000 dilution] and Synaptophysin [Abcam Cambridge, MA; 32127, 1:100,000 dilution]. Following incubation, the membranes were washed as previously described and re-blocked with 10% blotto. Each

membrane was incubated with the appropriate secondary antibody [Abcam, Cambridge, MA; 6728. 1:75,000 dilution; Abcam, Cambridge, MA; 6721, 1:200,000 dilution], then washed as previously described. Detection reagent (GE Healthcare, Cincinnati, OH; 45-001-173) was added to the membrane which was then exposed. Data analysis followed using GE ImageQuant Software.

The BDNF ELISA (7.8-500pg/mL) was completed using the BDNF E_{max}® Immunoassay System (Promega, Madison, WI; G7611), and validated by using brain and heart samples to verify cross reactivity was not observed. Briefly, the plate was incubated overnight at $4°C$ and the following day the wells were vigorously washed with tris-buffered saline and tween-20 (TBST) using an automated plate washer. Buffer was added to each well and the plate was incubated for one hour. The 6.7 mg/mL normalized supernatants were acid treated by adding 1N HCl until a pH below 3.0 was achieved, incubated for 15 minutes at room temperature, and using 1N NaOH were neutralized to a pH of approximately 7.6 and placed back on ice. Following incubation, the wells were washed as described. Samples were added to the plate in duplicate using 100µL of sample per well, and the plate was incubated for 2 hours with shaking at room temperature. After incubation, the plate was washed, and BDNF primary antibody (1:50) was added to each well, followed by a 2 hour incubation. The plate was then washed as previously described. Horseradish Peroxidase (HRP) Conjugate was added to each well, followed by another 1-hour incubation with shaking and a repeated washing. After washing, 3,3,5,5- tetramethylbenzidine (TMB) One solution was added to each well, then 1N HCL was placed into each well, and the plate was immediately read on the spectrophotometer at a wavelength of 450nM. Data was analyzed using the SoftMax Pro 5.2 software.

Localization of BDNF and trkB mRNA was performed using *in-situ* hybridization as described by Hicks et al. (1998). Briefly, the unperfused slide-mounted sections were pretreated by drying them using cold air. The slides were immersed in 4% paraformaldehyde for 10 minutes, washed twice for 5 min in 0.1M phosphate buffer saline (PBS), washed twice for 5 min in 0.2% PBS/Glycine, washed again in 0.1M PBS, and then placed in 0.25% acetic anhydride/0.1M triethanolamine for 10 minutes. The slides were then dehydrated, delipidated, and air dried. The BDNF and trkB cRNA probes (Kindly provided by Christine Gall, University of California; Irvine and Kathryn Albers, University of Pittsburgh) were prepared using in vitro transcription from a linearized cDNA construct with T3 polymerase in the presence of ^{35}S-UTP (NEG039H, Perkin Elmer). Hybridization was performed at 60°C for 18-24 hours. Following hybridization, slides were washed with 4x saline-sodium citrate (SSC) + 10mM sodium thiosulfate at 37°C twice for 30 minutes each, then incubated in ribonuclease A for 30 minutes at 45°C. Slides were washed in 2X SSC and 0.5X SSC two times each at 37°C for 20 minutes, and 0.1X SSC at 37°C for 30 minutes. Sections were dipped in dH_2O followed by 95% EtOH, air-dried and loaded into cassettes with β-Max Hyperfilm (Amersham). The films were exposed at room temperature for 10 days for BDNF and 7 days for trkB in order to generate film autoradiograms. Following development, films were dipped in GPX developer and fixer (Kodak), air dried and then exposed in light-tight boxes at 4°C for 2-4 weeks. Slides were analyzed using a Nikon Optiphot-2 microscope and Scion software (NIH). Analysis compared the density of hybridization for BDNF mRNA in the Piriform Cortex, Prefrontal Cingulate, Dentate Gyrus and CA3 subfields. Background hybridization from the proximal tissue was subtracted from the hybridization in the hippocampal subfields to obtain corrected

optical density (OD) measurements. Gray scale values were calculated by taking area of the region of interest into account as well.

3.7 Statistical Analysis
All results are expressed as mean ± standard error (SE), unless depicted differently in the figures. The data were tested for normality and equal variance using the Kolmogorow-Smirnov test and F-test, respectively. The significance level for all tests was 0.05. A one-way analysis of variance was used to compare groups ST-TBI, EE-TBI, and Sham. Post-hoc paired comparisons of groups used Tukey simultaneous confidence intervals with a 0.05 experimentwise error level.

4.0   RESULTS

One-way analysis of variance (ANOVA) was performed for corticosterone levels at baseline, basal, and stressed blood draws. There was not a significant difference among the groups at baseline, or following stress. At basal, when housing conditions were the only difference among groups, post-hoc analysis revealed that the EE-TBI group had significantly higher corticosterone levels (p=0.0029) than the ST-TBI or Sham groups (Figure 2). This result demonstrates that exposing animals to an enriched environment can elevate basal HPA axis activity.
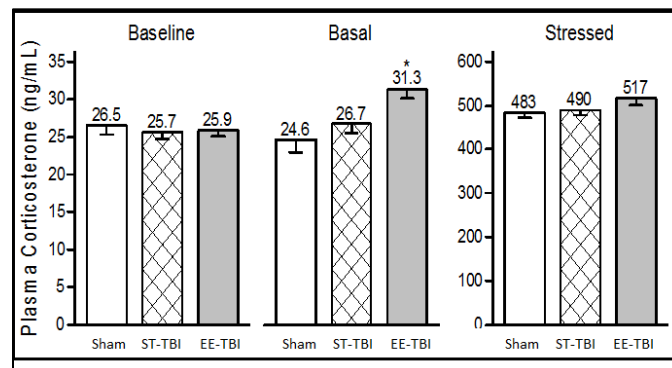

Figure 2: Corticosterone Results. Data show that during the basal blood draw, EE-TBI rodents had a significantly elevated Corticosterone response compared to the other groups.

Both the CA3 region of the hippocampus and perilesional area of the prefrontal cortex were analyzed for MAP2 and synaptophysin. A one-way ANOVA was performed for the corrected gray scale values for each combination of protein and location. No significant changes were observed.

The differences in the quantity of BDNF in the hippocampus among the varying conditions were minimal. BDNF protein concentration (pg/mL) did not show a significant difference among the groups when observed during the chronic stage of TBI.

Analysis of the film autoradiograms revealed slight elevations in BDNF mRNA for the EE-TBI group compared to ST-TBI; however no statistically significant differences in any of the measured brain regions among the groups were detected. There was however, a significant difference found (p=0.0067) in the Dentate Gyrus (DG) for trkB mRNA (Figure 3). The Tukey

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procedure revealed Sham was significantly higher than ST-TBI however; it did not show significant differences between Sham and EE-TBI trkB levels for this same region.
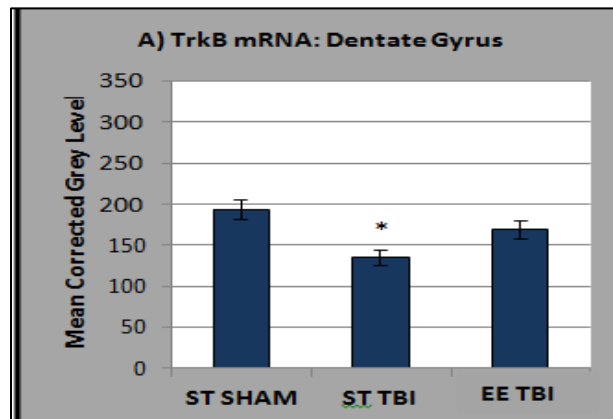

Figure 3: TrkB mRNA in Dentate Gyrus. Results show that the ST-TBI group has a significantly lower trkB mRNA response compared to the Sham Group.

Using the corrected grey scale values from the densitometric measurements, it was determined that neither housing conditions nor injury significantly alter the levels of BDNF mRNA when observed 33 days post- injury.

5.0   DISCUSSION

The current research sought to establish a foundational understanding of pre-injury EE effects on neuroprotective proteins during chronic stage TBI. Based on the positive behavioral results acquired by pre-enrichment exposure in the work by Johnson et al. (2012), it was hypothesized the EE before injury would provide protection against the functional deficits of chronic stage TBI due to elevated levels of particular neuroprotective biomarkers. Results showed significant differences in the BDNF receptor (trkB) and basal corticosterone levels, indicating some neuroprotective tendencies. However, significant differences in the other chosen biomarkers were not detected, indicating that these proteins may have played a role in an earlier stage of injury, and different biomarker options for the chronic stage should be explored in the future.

In the current research, it was hypothesized that EE applied prior to injury would cause elevated levels of BDNF and other biomarkers which would serve as neuroprotective mechanisms by remaining elevated through the chronic stage of TBI. Recent work by Johnson et al. (2012) illustrated that pre-enrichment improved performance on the Morris Water Maze task compared to standard housed animals during chronic stage TBI, indicating that EE provided neuroprotective tendencies possibly due to elevated levels of particular biomarkers which are involved in learning and memory. While our results did not validate these findings statistically, it was determined that EE-TBI BDNF mRNA levels were slightly elevated compared to ST-TBI animals in the hippocampus, which may have accounted for some of these neuroprotective measures following injury. Our results are supported by previous research by Gobbo et al. (2005) who found no significant differences in BDNF protein levels between post-enriched and standard housed injured animals at 3 weeks post-injury; however, BDNF levels were

slightly elevated in comparison to the standard housed animals. In a similar study by Bindu et al. (2007) the effects on BDNF levels 3 weeks post-injury showed that animals enriched after TBI experienced enhanced dendritic morphology and spine density, but the levels of BDNF remained insignificant between housing conditions.   These findings are contradictive of work looking strictly at chronic levels of BDNF mRNA following CCI (Griesbach et al., 2009). This author found that BDNF mRNA levels were significantly lower in the hippocampus compared to sham animals at 21 days post-injury, it should be noted however; that their study did not include an enrichment paradigm, providing further evidence that EE applied pre-injury may positively influence neuroprotective biomarkers during various stages of TBI. These studies demonstrate the positive effect EE may have on neuroprotection by elevating BDNF (possibly more significantly during other stages of TBI) and enhancing neuronal morphology when applied after injury. These same protective trends may have occurred in this study.

Other studies showing significant BDNF protein and mRNA elevations during the acute and sub-acute stages reveal it promoted cell survival (Hicks et al., 1997), hippocampal homeostasis (Marmigere at al., 2003) and generation of long-term potentiation (LTP) (Hicks et al., 1999), which could have led to the positive results Johnson et al. saw during their chronic behavioral testing. Capturing the trends in BDNF mRNA and protein levels before injury and during the acute and chronic stages of TBI may provide a better understanding of the mechanisms involved in enhancing physiological and cognitive outcomes following TBI using enrichment, and provide insight to how this biomarker influences the chronic stage of injury even though it is not significantly elevated at that point. By recognizing various protective biomarkers and optimizing their neuroprotective tendencies, it may lead to methods which will alleviate some of the detrimental effects caused by such a life-changing event.

Although significant changes in BDNF protein levels were not detected in this study, it was determined that trkB levels were reduced significantly in the dentate gyrus (DG) for the ST-TBI group compared to the Shams. During the acute stage of injury, Hicks et al. (1999) observed elevated levels of trkB in the DG for injured animals. It was determined that these levels began to decrease during the sub-acute stage due to cell dystrophy and inflammatory processes, which explains decreases seen in the current research. Decreases in the levels of trkB receptors may account for the behavioral deficits Johnson et al. (2012) observed in the ST-TBI group. A reduction in receptors would limit BDNF binding sites and thus, result in a lack of neuroprotective benefits. It should be noted that the EE-TBI trkB levels were not as diminished as the ST-TBI group during the chronic stage of injury, suggesting EE applied before TBI provided some neuroprotection against cell death in the dentate gyrus.

While BDNF was the main biomarker of interest in this study, other proteins were observed due to their roles in neuroprotection and improved cognitive performance. MAP2 is a protein which serves as a marker of dendritic density and has been associated with dendritic plasticity following learning (Derksen et al., 2007) while synaptophysin expression is related to synaptogenesis and has been associated to functional recovery after injury (Khan et al., 2011). In this study, no significant changes in the hippocampus or prefrontal cortex were observed during the chronic stage of TBI for MAP2 or synaptophysin. This finding is surprising based on research looking at skilled exercise training (which the EE-TBI group performed prior to injury) showing hippocampal neurogenesis (van Praag et al., 1999) and synaptogenesis (Kleim et al., 1998), however these studies observed

these biomarkers shortly after training occurred. A 2001 study did report similar findings to the current research however, showing that during the chronic stage of TBI both post-injury enriched and non-enriched animals did not have significantly elevated MAP2 or synaptophysin levels compared to baseline (Passineau et al., 2001). Therefore, not observing changes in the current work may indicate these levels were affected during the acute and sub-acute stages of TBI, providing beneficial effects before returning to control levels. Future studies are required to understand how enrichment can influence the neuroprotective benefits of MAP2 and synaptophysin following TBI, however in the current study it was determined these biomarkers do not remain elevated in the hippocampus during the chronic stage of injury.

Chronic and baseline levels of corticosterone, a marker of HPA activity, were not found to be significantly different between groups in the current study. However, basal corticosterone levels were elevated in enriched animals compared to standard housed rodents. This elevation is supported by Benaroya et al. (2004) and is thought to be due to the constant stressors (multiple cage mates, motor skills training, novel stimuli) that the enriched rodents are exposed to compared to standard housed animals. Long term exposure to glucocorticoids, such as corticosteroid, has been shown to cause cognitive impairments, however short term exposure to low levels may enhance cognitive performance due to a more adaptive HPA axis response (Larsson et al., 2002). By observing the positive behavioral outcomes that Johnson et al. (2012) revealed and correlating the elevated basal levels from this study, it is clear that EE has a positive influence on the stress response when applied before TBI.

6.0   CONCLUSION

It was hypothesized that EE, when applied prior to injury, would provide protection against the detrimental effects of TBI and that this protection would be accompanied by elevated levels of particular biomarkers during the chronic stage of injury. From the results, it is clear that the selected biomarkers play a role in neuroprotection when enrichment is applied pre-injury, but these positive effects are not detectable during the chronic stage of TBI. This work demonstrates that a signature of chronic TBI is not distinguishable with the chosen biomarkers and future research should focus on their effects prior to injury as well as during the acute and sub-acute stage of TBI. This will allow the neuroprotective mechanism to be established, which will provide a way forward for developing the best mitigation strategies for those suffering the detriments of a traumatic brain injury

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Appendix A

ACRONYMS

CCI	controlled cortical impact
EE	environmental enrichment
GC	glucocorticoid
HPA	hypothalamic-pituitary-adrenal
TBI	traumatic brain injury